encompassing 132,538 newborns required a second DBS from 1093 newborns (0.82% of total) when the cut-off value was set at 25% of normal mean AαGlu activity [4]. Most of these recalls might have been c.[1726A; 2065A] homozygotes. After publication of the Taiwanese study, we tried to improve the feasibility of the screening procedure by adopting the assay conditions described by Chien et al. [4]. Forty DBSs from 12 patients and 28 c.[1726A; 2065A] homozygotes were subjected to the screening procedure with the following cut-off values; the AαGlu activity, less than 8% of normal mean; the ratio of neutral over acid α-glucosidase activities, more than 60; and the percentage inhibition of total α-glucosidase activity by acarbose, more than 80% [4]. Using the combination of these criteria, none of the c.[1726A; 2065A] homozygotes was identified as false positive, but 2 of the 12 patients were misdiagnosed (unpublished data). The use of a different substrate coupled to a different assay procedure (e.g., the newly developed substrates for tandem mass spectrometry) might solve the problem [10–13]. Tandem mass spectrometry worked remarkably well in a pilot study in 10,279 Austrian newborns. The calculated recall rate would have been only 0.039% [5]. But, it must be emphasized that the great majority of Austrians is Caucasian, and that the frequency of c.[1726A; 2065A] homozygotes in that population is probably very low.

As it stands, our method based on the measurement of AαGlu activity in DBSs using 4MU-αGlc in the presence of 3.0 μmol/L acarbose detects 1.5% of Japanese newborns as potential patients at a cut-off value of 4.0 pmol/h/disk without false negative outcomes. But, at lower cut-off value some patients will be missed (Tables 2 and 3). We have demonstrated the feasibility of obtaining *GAA* genetic information from the DNA that remains on the DBSs, but the application is not sufficient as second tier test. If an activity of less than 3 pmol/h/disk is found in an individual with any diplotype other than c.[1726A; 2065A] homozygote, this individual is very likely to have GSDII. However, a c.[1726A; 2065A] homozygote with low activity remains to be diagnosed.

Notably, there were three c.[1726A; 2065A] homozygotes among the 18 proven patients. Although the sample size is small, it seems that the frequency of the c.[1726A; 2065A] allele is higher in the patient (42%) than in the control population (19%), which is suggestive for a founder effect. This hypothesis can be investigated by *GAA* sequence analysis of the 12 patients that are either homozygote or heterozygote for c.[1726A; 2065A]. They are then expected to share a common pathogenic mutation besides the linked c.1726G>A and c.2065G>A SNPs. Gene sequencing is in progress to clarify this possibility. Interestingly, c.1935C>A leading to amino acid substitution D645E is the most common pathogenic mutation in the Southeastern part of China and in Taiwan. This mutation was first reported by Shieh and Lin [28] to be linked to c.2065G>A, whereas linkage to c.1726G>A was not investigated at that time. Recently, Wan et al. reported that they could not find any specific polymorphism that links to the pathogenic c.1935C>A mutation in the same Chinese population [29]. This would mark position c.1935 as a mutational hotspot, although c.1935C>A is not very common in Japan among patients with GSDII (6.8% of the mutant alleles) [30,31].

In conclusion, our findings illustrate that homozygosity for the c.[1726A; 2065A] allele, resulting in "pseudodeficiency" of AαGlu, complicates newborn screening for GSDII in the Japanese and other Asian populations. Our findings also suggest that one or more pathogenic mutations are associated with this allele. Further investigations are required to optimize the selectivity of the newborn screening procedure and to minimize the number of cases that have to be recalled for second or third tier testing.

Table 3
Diagnostic property of newborn screening with different cut-off values on DBSs from 715 controls and 18 patients in Japanese populations.

Cut-off value (pmol/h/disk)	Normal mean activity (%)	False positive (%)	False negative (%)
10.0	34.0	39/715 (5.5)	0/18 (0)
9.0	30.6	31/715 (4.3)	0/18 (0)
8.0	27.2	30/715 (4.2)	0/18 (0)
7.0	23.8	28/715 (3.9)	0/18 (0)
6.0	20.4	26/715 (3.6)	0/18 (0)
5.0	17.0	18/715 (2.5)	0/18 (0)
4.0	13.6	11/715 (1.5)	0/18 (0)
3.0	10.2	4/715 (0.6)	0/18 (0)
2.0	6.8	2/715 (0.3)	1/18 (5.5)

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References

[1] N.A. Chamoles, G. Niizawa, M. Blanco, D. Gaggioli, C. Casentini, Glycogen storage disease type II: enzymatic screening in dried blood spots on filter paper, Clin. Chim. Acta 347 (2004) 97–102.
[2] M. Spada, S. Pagliardini, M. Yasuda, T. Tukel, G. Thiagarajan, H. Sakuraba, A. Ponzone, R.J. Desnick, High incidence of later-onset Fabry disease revealed by newborn screening, Am. J. Hum. Genet. 79 (2006) 31–40.
[3] H. Zhang, H. Kallwass, S.P. Young, C. Carr, J. Dai, P.S. Kishnani, D.S. Millington, J. Keutzer, Y.T. Chen, D. Bali, Comparison of maltose and acarbose as inhibitors of maltase-glucoamylase activity in assaying acid alpha-glucosidase activity in dried blood spots for the diagnosis of infantile Pompe disease, Genet. Med. 8 (2006) 302–306.
[4] Y.H. Chien, S.C. Chiang, X.K. Zhang, J. Keutzer, N.C. Lee, A.C. Huang, C.A. Chen, M.H. Wu, P.H. Huang, F.J. Tsai, Y.T. Chen, W.L. Hwu, Early detection of Pompe disease by newborn screening is feasible: results from the Taiwan screening program, Pediatrics 122 (2008) e39–45.
[5] A. Dajnoki, A. Muhl, G. Fekete, J. Keutzer, J. Orsini, V. Dejesus, X.K. Zhang, O.A. Bodamer, Newborn screening for Pompe disease by measuring acid alpha-glucosidase activity using tandem mass spectrometry, Clin. Chem. 54 (2008) 1624–1629.
[6] K. Umapathysivam, A.M. Whittle, E. Ranieri, C. Bindloss, E.M. Ravenscroft, O.P. van Diggelen, J.J. Hopwood, P.J. Meikle, Determination of acid alpha-glucosidase protein: evaluation as a screening marker for Pompe disease and other lysosomal storage disorders, Clin. Chem. 46 (2000) 1318–1325.
[7] K. Umapathysivam, J.J. Hopwood, P.J. Meikle, Determination of acid alpha-glucosidase activity in blood spots as a diagnostic test for Pompe disease, Clin. Chem. 47 (2001) 1378–1383.
[8] P.J. Meikle, E. Ranieri, H. Simonsen, T. Rozaklis, S.L. Ramsay, P.D. Whitfield, M. Fuller, E. Christensen, F. Skovby, J.J. Hopwood, Newborn screening for lysosomal storage disorders: clinical evaluation of a two-tier strategy, Pediatrics 114 (2004) 909–916.
[9] P.J. Meikle, D.J. Grasby, C.J. Dean, D.L. Lang, M. Bockmann, A.M. Whittle, M.J. Fietz, H. Simonsen, M. Fuller, D.A. Brooks, J.J. Hopwood, Newborn screening for lysosomal storage disorders, Mol. Genet. Metab. 88 (2006) 307–314.
[10] Y. Li, C.R. Scott, N.A. Chamoles, A. Ghavami, B.M. Pinto, F. Turecek, M.H. Gelb, Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening, Clin. Chem. 50 (2004) 1785–1796.
[11] M.H. Gelb, F. Turecek, C.R. Scott, N.A. Chamoles, Direct multiplex assay of enzymes in dried blood spots by tandem mass spectrometry for the newborn screening of lysosomal storage disorders, J. Inherit. Metab. Dis. 29 (2006) 397–404.
[12] X.K. Zhang, C.S. Elbin, W.L. Chuang, S.K. Cooper, C.A. Marashio, C. Beauregard, J.M. Keutzer, Multiplex enzyme assay screening of dried blood spots for lysosomal storage disorders by using tandem mass spectrometry, Clin. Chem. 54 (2008) 1725–1728.
[13] V.R. De Jesus, X.K. Zhang, J. Keutzer, O.A. Bodamer, A. Muhl, J.J. Orsini, M. Caggana, R.F. Vogt, W.H. Hannon, Development and evaluation of quality control dried blood spot materials in newborn screening for lysosomal storage disorders, Clin. Chem. 55 (2009) 158–164.
[14] H. Van den Hout, A.J. Reuser, A.G. Vulto, M.C. Loonen, A. Cromme-Dijkhuis, A.T. Van der Ploeg, Recombinant human alpha-glucosidase from rabbit milk in Pompe patients, Lancet 356 (2000) 397–398.
[15] L. Klinge, V. Straub, U. Neudorf, T. Voit, Enzyme replacement therapy in classical infantile Pompe disease: results of a ten-month follow-up study, Neuropediatrics 36 (2005) 6–11.

[16] P.S. Kishnani, D. Corzo, M. Nicolino, B. Byrne, H. Mandel, W.L. Hwu, N. Leslie, J. Levine, C. Spencer, M. McDonald, J. Li, J. Dumontier, M. Halberthal, Y.H. Chien, R. Hopkin, S. Vijayaraghavan, D. Gruskin, D. Bartholomew, A. van der Ploeg, J.P. Clancy, R. Parini, G. Morin, M. Beck, G.S. De la Gastine, M. Jokic, B. Thurberg, S. Richards, D. Bali, M. Davison, M.A. Worden, Y.T. Chen, J.E. Wraith, Recombinant human acid [alpha]-glucosidase: major clinical benefits in infantile-onset Pompe disease, Neurology 68 (2007) 99–109.
[17] M. Rossi, G. Parenti, R. Della Casa, A. Romano, G. Mansi, T. Agovino, F. Rosapepe, C. Vosa, E. Del Giudice, G. Andria, Long-term enzyme replacement therapy for Pompe disease with recombinant human alpha-glucosidase derived from Chinese hamster ovary cells, J. Child Neurol. 22 (2007) 565–573.
[18] C.I. van Capelle, L.P. Winkel, M.L. Hagemans, S.K. Shapira, W.F. Arts, P.A. van Doorn, W.C. Hop, A.J. Reuser, A.T. van der Ploeg, Eight years experience with enzyme replacement therapy in two children and one adult with Pompe disease, Neuromuscul. Disord. 18 (2008) 447–452.
[19] L.P. Winkel, J.M. Van den Hout, J.H. Kamphoven, J.A. Disseldorp, M. Remmerswaal, W.F. Arts, M.C. Loonen, A.G. Vulto, P.A. Van Doorn, G. De Jong, W. Hop, G.P. Smit, S.K. Shapira, M.A. Boer, O.P. van Diggelen, A.J. Reuser, A.T. Van der Ploeg, Enzyme replacement therapy in late-onset Pompe's disease: a three-year follow-up, Ann. Neurol. 55 (2004) 495–502.
[20] T. Merk, T. Wibmer, C. Schumann, S. Kruger, Glycogen storage disease type II (Pompe disease)–influence of enzyme replacement therapy in adults, Eur. J. Neurol. 16 (2009) 274–277.
[21] A.T. van der Ploeg, A.J. Reuser, Pompe's disease, Lancet 372 (2008) 1342–1353.
[22] T. Okumiya, J.L. Keulemans, M.A. Kroos, N.M. Van der Beek, M.A. Boer, H. Takeuchi, O.P. Van Diggelen, A.J. Reuser, A new diagnostic assay for glycogen storage disease type II in mixed leukocytes, Mol. Genet. Metab. 88 (2006) 22–28.
[23] Y. Tajima, F. Matsuzawa, S. Aikawa, T. Okumiya, M. Yoshimizu, T. Tsukimura, M. Ikekita, S. Tsujino, A. Tsuji, T. Edmunds, H. Sakuraba, Structural, biochemical studies on Pompe disease and a "pseudodeficiency of acid alpha-glucosidase", J. Hum. Genet. 52 (2007) 898–906.
[24] M.A. Kroos, R.A. Mullaart, L. Van Vliet, R.J. Pomponio, H. Amartino, E.H. Kolodny, G.M. Pastores, R.A. Wevers, A.T. Van der Ploeg, D.J. Halley, A.J. Reuser, p.[G576S; E689K]: pathogenic combination or polymorphism in Pompe disease?, Eur J. Hum. Genet. 16 (2008) 875–879.
[25] Y. Suzuki, A. Tsuji, K. Omura, G. Nakamura, S. Awa, M. Kroos, A.J. Reuser, Km mutant of acid alpha-glucosidase in a case of cardiomyopathy without signs of skeletal muscle involvement, Clin. Genet. 33 (1988) 376–385.
[26] B.E. Nickel, P.J. McAlpine, Extension of human acid alpha-glucosidase polymorphism by isoelectric focusing in polyacrylamide gel, Ann. Hum. Genet. 46 (1982) 97–103.
[27] M.L. Huie, M. Menaker, P.J. McAlpine, R. Hirschhorn, Identification of an E689K substitution as the molecular basis of the human acid alpha-glucosidase type 4 allozyme (GAA 4), Ann. Hum. Genet. 60 (1996) 365–368.
[28] J.J. Shieh, C.Y. Lin, Frequent mutation in Chinese patients with infantile type of GSD II in Taiwan: evidence for a founder effect, Hum. Mutat. 11 (1998) 306–312.
[29] L. Wan, C.C. Lee, C.M. Hsu, W.L. Hwu, C.C. Yang, C.H. Tsai, F.J. Tsai, Identification of eight novel mutations of the acid α-glucosidase gene causing the infantile or juvenile form of glycogen storage disease type II, J. Neurol. 255 (2008) 831–838.
[30] S. Tsujino, M. Huie, N. Kanazawa, H. Sugie, Y. Goto, M. Kawai, I. Nonaka, R. Hirschhorn, N. Sakuragawa, Frequent mutations in Japanese patients with acid maltase deficiency, Neuromuscul. Disord. 10 (2000) 599–603.
[31] J.R. Pipo, J.H. Feng, T. Yamamoto, Y. Ohsaki, E. Nanba, S. Tsujino, N. Sakuragawa, F. Martiniuk, H. Ninomiya, A. Oka, K. Ohno, New GAA mutations in Japanese patients with GSDII (Pompe disease), Pediatr. Neurol. 29 (2003) 284–287.

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Original article

Intracerebral cell transplantation therapy for murine GM1 gangliosidosis

Tomo Sawada [a], Akemi Tanaka [a,*], Katsumi Higaki [c], Ayumi Takamura [c], Eiji Nanba [c], Toshiyuki Seto [a,e], Mitsuyo Maeda [b], Etsuko Yamaguchi [a], Junichiro Matsuda [d], Tunekazu Yamano [a]

[a] *Department of Pediatrics, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan*
[b] *Department of Neurobiology and Anatomy, Osaka City University Graduate School of Medicine, Osaka, Japan*
[c] *Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University, Yonago, Japan*
[d] *Laboratory of Experimental Animal Models, Division of Bioresources, National Institute of Biomedical Innovation, Osaka, Japan*
[e] *Department of Pediatrics, Fujiidera City Hospital, Fujiidera, Japan*

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Abstract

We performed a cell transplantation study to treat the brain involvement in lysosomal storage diseases. We used acid β-galactosidase knock-out mice (BKO) from C57BL/6 as recipients. To minimize immune responses, we used cells derived from transgenic mice of C57BL/6 overexpressing the normal human β-galactosidase. Fetal brain cells (FBC), bone marrow-derived mesenchymal stem cells (MSC), and mixed FBC and MSC cells were prepared and injected into the ventricle of newborn BKO mouse brain. The mice were examined at 1, 2, 4, and 8 weeks and 6 months after injection. In each experiment, the injected cells migrated into the whole brain effectively and survived for at least 8 weeks. Decrease in ganglioside GM1 level was also observed. FBC could survive for 6 months in recipient brain. However, the number of transplanted FBC decreased. In the brains of MSC- or mixed cell-treated mice, no grafted cells could be found at 6 months. To achieve sufficient long-term effects on the brain, a method of steering the immune response away from cytotoxic responses or of inducing tolerance to the products of therapeutic genes must be developed.

Keywords: GM1-gangliosidosis; Cell transplantation; Fetal brain cell; Mesenchymal stem cell

1. Introduction

Enzyme replacement therapy (ERT), hematopoeitic stem cell transplantation (HSCT), and gene transfer have been studied in animals and in humans with lysosomal storage disease (LSD). ERT is now available clinically for Gaucher disease, Fabry disease, Pompe disease, and MPS I, II, and VI in many countries, and has been successful in visceral organs. HSCT is also effective against the somatic involvements in Gaucher disease and MPS I, II, and VI. However, HSCT exhibits little efficacy in conditions such as Fabry disease and Pompe disease, when enzyme secretion from donor cells is poor or the uptake of enzyme proteins by the affected host cells is inadequate. In addition, efficacy in individual organs differs markedly, in both ERT and HSCT, depending on accessibility of blood flow and the density of mannose-6-phosphate receptors. Neither HSCT nor ERT exhibits efficacy against the brain involvement in Gaucher or MPSs because of the poor access due to the blood–brain barrier.

Many experimental studies have been carried out, involving methods such as gene therapy [1–5], cell

* Corresponding author. Tel.: +81 6 6645 3816; fax: +81 6 6636 8737.
E-mail address: akemi-chan@med.osaka-cu.ac.jp (A. Tanaka).

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therapy [6–9], or intrathecal administration of enzymes [10,11], for treatment of the brain in LSDs. Such treatments were able to overcome the blood–brain barrier to access brain tissue and exhibit considerable efficacy in brain. However, it is difficult to maintain such efficacy for long periods of time. Repetition of these treatments is not practical because intracranial administration is required for them. On the other hand, the usefulness of intravenous administration is limited because of the blood–brain barrier, except in newborn mice which have an immature barrier. It has been reported that intravenous administration of extremely high doses of enzymes [12–14] or of enzymes that remain in the circulation for long periods [15,16] yielded slight passage through the blood–brain barrier, though with increase in the risk of immune response.

Oral administration of small molecules would be a good and convenient method of treatment of the brain for prolonged periods, such as substrate reduction therapy with *N*-butyldeoxynojirimycin or *N*-butyldeoxygalactonojirimycin for glycosphingolipidoses [17–19] or genistein for mucopolysaccharidoses [20], and chemical chaperone therapy for Fabry disease [21] or GM1-gangliosidosis [22]. However, the efficacy of substrate reduction therapies has thus far been quite limited, and chemical chaperone therapies are not applicable for every type of gene mutation.

GM1 gangliosidosis is an LSD and a progressive neurological disease in humans caused by a genetic defect of lysosomal acid β-galactosidase, which hydrolyses the terminal β-galactosidic residue of ganglioside GM1 and other glycoconjugates. The defects in β-galactosidase activity result in accumulation of ganglioside GM1 in various organs, especially the brain, causing progressive neurodegeneration. In our previous study [2], we injected recombinant adenovirus encoding mouse β-galactosidase cDNA intravenously in β-galactosidase-deficient newborn mice, and showed that vector-mediated β-galactosidase-producing brain cells could reduce ganglioside GM1 accumulation. We showed that β-galactosidase enzyme protein could be secreted as well as taken up by the brain cells and function effectively. However, the efficacy obtained was transient. If sufficient amounts of the defective enzyme could be permanently secreted by cells in the brain, injury of the brain could be prevented. To examine the possibility of long-term cell treatment of the brain in LSDs, we carried out a transplantation experiment in the brain of a GM1-gangliosidosis mouse model (acid β-galactosidase knock-out mouse) using fetal brain cells (FBC) and mesenchymal stem cells (MSC) from bone marrow. These cells used for transplantation were derived from mice of the same genetic background as recipient mice except for possession of the human β-galactosidase gene.

2. Materials and methods

2.1. Knock-out and transgenic mice

A mouse model of GM1 gangliosidosis (BKO mouse) was generated by targeting of the β-galactosidase gene at exon 15 in ES cells as previously described [23]. Newborn mice were obtained by mating heterozygous female mice with homozygous male mice. Identification of newborn mutants was accomplished by quantitative analysis of β-galactosidase activity in tail tip homogenates on the day of birth. Mice with high β-galactosidase activity (TG mice) [24] were generated by introducing the human β-galactosidase gene as a transgene in ES cells obtained from the BKO mouse, which has several copies of the human β-galactosidase gene without the mouse β-galactosidase background. Age-matched wild-type mice of C57BL/6 strain were used as a control.

2.2. Cell preparations for transplantation

Cultured mesenchymal stem cells (MSC) were obtained from the bone marrow of the tibias and femurs of 5–8 month-old TG mice according to the method of Meirelles et al. [25] with some modifications. Dulbecco's modified Eagle's medium (DMEM: Sigma Chemical Co., St Louis, MO) containing 10% fetal bovine serum (Medical and Biological Laboratories, Nagoya, Japan) was used for culture.

Fetal brain cells (FBC) were obtained from the fetal cerebral cortex of TG mice at 13 days of gestation according to the method of Meberg and Miller [26]. The brain tissue was disrupted in a Pasteur glass pipette by gentle stroking several times (uncultured FBC), and then cultured for 4 h in Neurobasal medium (Invitrogen, Carlsbad, CA, No. 12348-07) containing 2 mM glutamine and 10% FBS, followed by two days in Neurobasal medium containing 2 mM glutamine and B27 supplement (Invitrogen, No. 14175-095) (cultured FBC).

2.3. Transplantation of cells into newborn mouse brain

Each BKO mouse received a single injection of 0.5–1.0 $\times 10^5$ of the cells prepared as described above in the right cerebral ventricle from 24 to 48 hours after birth. Study groups were as follows: uncultured FBC ($n = 18$), cultured FBC ($n = 10$), MSC ($n = 17$), and mixed MSC and FBC (1:1) ($n = 15$). Mice of each experimental group were divided into three subgroups for X-gal staining, β-galactosidase assay and ganglioside GM1 analysis. Mice were examined at one, two, four, and eight weeks and 6 months after injection as shown in Table 1.

For biochemical analysis, mice were anesthetized with diethylether and the blood was washed out with normal saline by perfusion through the heart, and the

brains were removed and kept at −80 °C until use. For histological studies, the brains were fixed by perfusion through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PB) for 20 min., after washing out the blood with normal saline. To obtain frozen sections, the brains were placed in 0.1 M phosphate buffer pH 7.4 containing 30% sucrose, and frozen in liquid nitrogen.

All surgical and care procedures were carried out in accordance with the Guidelines for Use and Care of Experimental Animals approved by the Animal Committee of Osaka City University School of Medicine.

2.4. X-Gal staining

Frozen sections (16 μm thick) were reacted with X-gal using the β-gal staining Kit (Invitrogen Corp., Carlsbad, CA) to visualize β-galactosidase activity.

2.5. β-Galactosidase assay

β-Galactosidase activity was analyzed in the tissue homogenate with the artificial substrate 2 mM 4-methylumbelliferyl β-galactoside at pH 4.0 in 0.1 M sodium citrate-phosphate buffer according to the method described by Suzuki [27]. Protein was analyzed using the Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA) with the method of Bradford [28].

2.6. Analysis of ganglioside GM1

Amounts of ganglioside GM1 were measured by immunoblot assay using anti-GM1 ganglioside monoclonal antibody (Code: 370685, Seikagaku Corp., Tokyo, Japan) by the method of Michikawa et al. [29] with some modifications.

Brain tissue cells were disrupted by sonication and solubilized in 20 mM Tris–HCl buffer pH 8.0 containing 137 mM NaCl, 10% glycerol, and a protease inhibitor cocktail (Complete, Mini, Cat No. 11836153001, Roche Diagnostics, Mannheim, Germany). Five micrograms of tissue protein was applied onto Trans-Blot Transfer Medium Pure Nitrocellulose Membrane (0.45 μm pore size, Code: 162-0117, Bio-Rad Laboratories) through the slots of a Bio-Dot SF Microfiltration Apparatus (Bio-Rad Laboratories). The membrane was reacted with anti-GM1 ganglioside monoclonal antibody diluted 1:500, after blocking with 5% skim milk in PBS solution for 1 h at room temperature, and then with horseradish peroxidase-linked anti-mouse IgG sheep antibody (Code: NA931, GE Healthcare UK Ltd., Buckinghamshire, UK) diluted 1:1,000. The washing solution used was 0.1 M Tris buffered saline pH 7.5 containing 0.1% Tween 20 (TTBS). Bound antibody was detected using ECL after reaction with ECL™ Western Blotting Detection Reagents (Code: RPN2209, GE Healthcare UK Ltd.) and visualized on X-ray film. Densitometric quantification of immunoreactive signal was performed using the Kodak Digital Science™ EDAS 120 system with 1D Image Analysis software (Eastman Kodak Company, NY). The values obtained were compared with those of quantification of histological immunoreactivity with Leica Control Software as previously described [30], and the same ratios were obtained among the samples (data not shown). The assay was performed three times and in duplicate for each sample independently, and mean values were calculated.

3. Results

3.1. X-Gal staining

Layered staining of the transplanted cells was observed over the entire ventricular surface on both sides of the cerebral hemispheres in treated mice at one week after injection (data not shown). Positive cells had spread into the brain tissue by two weeks (Fig. 1c and f) in the mice treated with cultured FBC ($n = 1$), uncultured FBC ($n = 1$), and MSC ($n = 2$) in the same amounts. The cells had spread further and had reached every part of the brain by 4 weeks in the mice of all experimental groups (Fig. 1d, g and i). Less positive cells were found in the mice treated with MSC ($n = 3$) or mixed MSC and FBC ($n = 3$) (Fig. 1g and i) than in the mice treated with cultured ($n = 3$) or uncultured FBC ($n = 3$) (Fig. 1d). The number of the X-Gal positive cells increased gradually until 4 weeks after injection in every experimental mouse. At 8 weeks after injection, positive cells still existed in the cultured FBC- ($n = 3$) and uncultured FBC-treated ($n = 3$) mice (Fig. 1e) in the same numbers with a similar distribution as at 4 weeks. However, a significant decrease in number of positive cells was found at 8 weeks in the mice treated with MSC ($n = 3$) or mixed MSC and FBC ($n = 3$) (Fig. 1h and j). In the mice treated with mixed MSC and FBC, positive cells existed in higher numbers in deep areas than in the mice treated with MSC alone. In the mice treated with cultured ($n = 2$) and uncultured FBC ($n = 2$), small numbers of positive cells with strong staining still existed in many parts of the brain, especially around the striatum and lateral globs pallidus (Fig. 1k and l), at 6 months after injection. No grafted cells were found in the mice treated with MSC ($n = 1$) or mixed MSC and FBC ($n = 1$) at 6 months. No significant differences were noted among the mice within each experimental group at each stage.

3.2. β-Galactosidase activity

The β-galactosidase activity in FBC and MSC derived from TG mice were 214.5–227.5 nmol/mg/h ($n = 4$) and 143.0–121.4 nmol/mg/h ($n = 3$), respec-

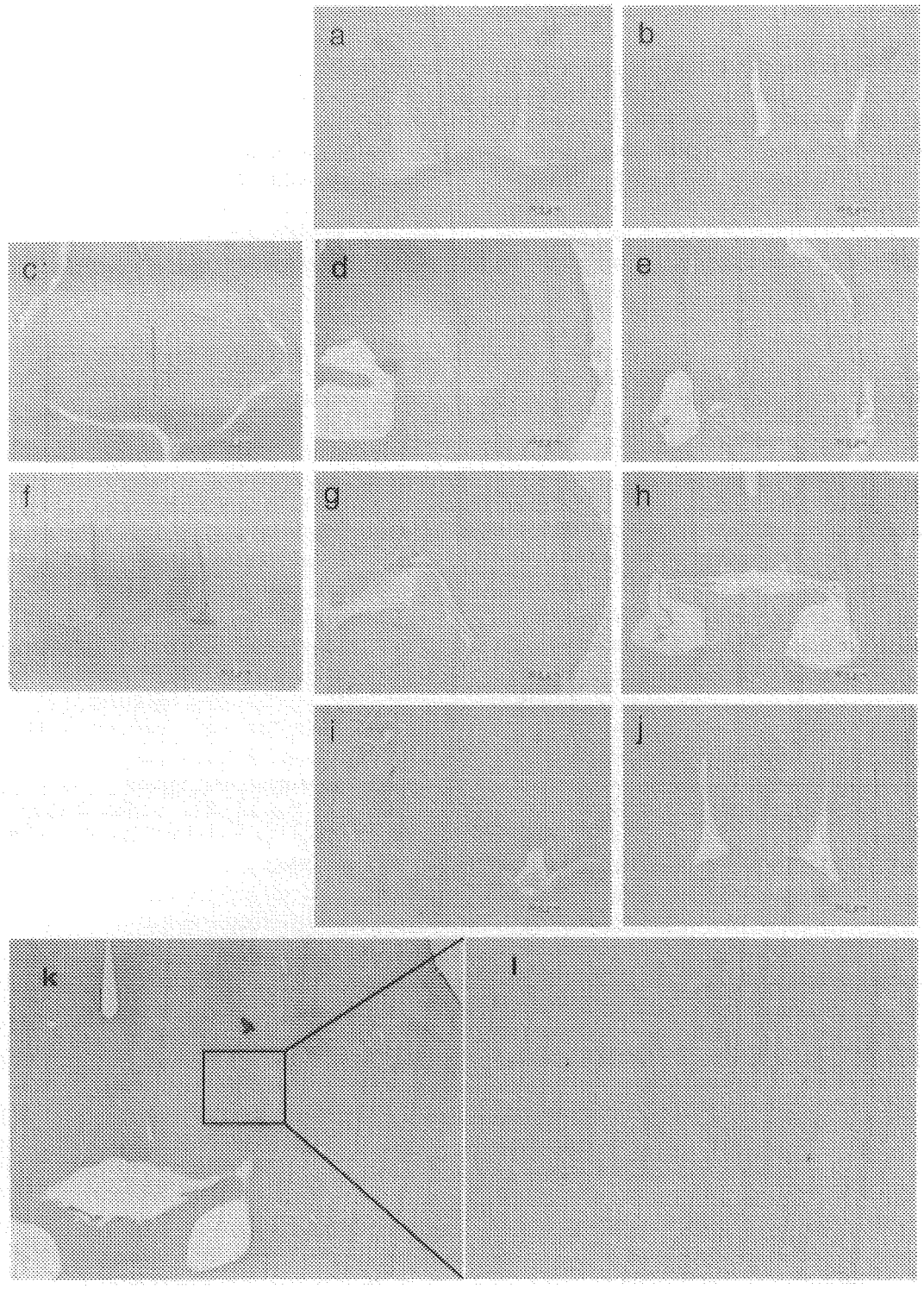

Fig. 1. X-Gal staining of brain coronal sections at +0.8 mm to −2.0 mm of bregma. (a and b) Non-treated BKO mouse at 4 and 8 weeks old, respectively; (c–e) Treated with FBC at 2, 4, and 8 weeks after injection; (f–h) Treated with MSC at 2, 4, and 8 weeks after injection; (i and j) Treated with mixed MSC and FBC at 4 and 8 weeks after injection; (k) FBC-treated brain at 6 months after injection; (l) Magnification of figure k. Positive cells had spread into the brain tissue by two weeks (c and f). The cells had spread further by 4 weeks (d, g and i). Less positive cells were found in the mice treated with MSC or mixed MSC and FBC (g and i) than in the mice treated with FBC (d). At 8 weeks, positive cells still existed in FBC-treated mouse (e) as at 4 weeks (d). A significant decrease in number of positive cells was found at 8 weeks in the mice treated with MSC (h) or mixed MSC and FBC (j). Strong positive staining cells still existed at 6 months in the brain of FBC-treated mouse (k and l).

tively, while the activity in FBC and in MSC derived from wild-type mice were 54.9–69.1 ($n = 2$) and 63.0 ($n = 1$), respectively.

The results of brain β-galactosidase activity in transplantation experiments are shown in Table 2. Increases in β-galactosidase activity were found in the brains of each experimental group at 4 weeks after injection. Activity in the FBC-treated mice was definitely increased at 4 weeks as well as at 8 weeks, while activity at 8 weeks in the MSC-treated mice and mixed MSC and FBC-treated mice was almost the same level as that in the untreated mice. These findings were consistent with those in the X-Gal staining study.

3.3. Immunoassay of ganglioside GM1

Immunoassay of accumulated ganglioside GM1 was performed for each mouse using anti-GM1 ganglioside monoclonal antibody. Values are ratios to the amounts in age-matched normal control mice. The results are shown in Fig. 2 and Tables 3. At 4 weeks after injection, remarkable decrease in ganglioside GM1 accumulation

Table 1
Mouse numbers used for each experiment.

Time after injection	1 week	2 weeks	4 weeks	8 weeks	6 months
	[X-Gal staining]				
Uncultured FBC	1	1	3	3	2
Cultured FBC	1	1	3	3	2
MSC		2	3	3	1
Mixed MSC and FBC			3	3	1
	[β-galactosidase activity]				
Uncultured FBC			2	2	
Cultured FBC					
MSC			2	2	
Mixed MSC and FBC			2	2	
	[Immunoblot assay of ganglioside GM1 amount]				
Uncultured FBC			1	1	1
Cultured FBC					
MSC			2	2	
Mixed MSC and FBC			2	2	

Table 2
β-Galactosidase activity.

	4 weeks	8 weeks
Age-matched normal control (mean ± SD)	197 ± 61 ($n = 7$)	159 ± 56 ($n = 7$)
Non-treated (mean ± SD)	4.38 ± 0.35 ($n = 5$)	4.10 ± 0.47 ($n = 5$)
Treated with uncultured FBC	Mouse 1	Mouse 7
	Rt: 6.65[a]	Rt: 4.94
	Lt: 5.31[a]	Lt: 6.03[a]
	Mouse 2	Mouse 8
	Rt: 7.36[a]	Rt: 5.58[a]
	Lt: 5.33[a]	Lt: 5.05[a]
Treated with MSC	Mouse 3	Mouse 9
	Rt: 6.30[a]	Rt: 4.13
	Lt: 5.95[a]	Lt: 3.67
	Mouse 4	Mouse 10
	Rt: 5.74[a]	Rt: 4.19
	Lt: 5.12[a]	Lt: 5.05[a]
Treated with mixed MSC and FBC	Mouse 5	Mouse 11
	Rt: 5.80[a]	4.13 (mix of both hemispheres)
	Lt: 5.40[a]	
	Mouse 6	Mouse 12
	Rt: 5.06	Rt: 4.85
	Lt: 4.52	Lt: 5.02

Values are in nmol/mg/h. Each sample was tested in duplicate and results are mean values. Rt, right hemisphere; Lt, left hemisphere.
[a] Increase of activity over mean + 2SD of non-treated mice.

Table 3
Immunoblot assay of ganglioside GM1 amount.

	4 weeks	8 weeks	6 months
Age-matched non-treated (range)	2.65–3.55 ($n = 3$)	4.98–5.28 ($n = 3$)	7.58 (n = 1)
Treated with uncultured FBC	Mouse I	Mouse VI	Mouse XI
	Rt: 1.42[a]	Rt: 2.30[a]	Rt: 6.18[b]
	Lt: 1.80[a]	Lt: 2.44[a]	Lt: 6.40[b]
Treated with MSC	Mouse II	Mouse VII	
	Rt: 1.82[a]	Rt: 5.30	
	Lt: 1.31[a]	Lt: 5.23	
	Mouse III	Mouse VIII	
	Rt: 1.40[a]	Rt: 4.40[b]	
	Lt: 1.34[a]	Lt: 4.73[b]	
Treated with mixed MSC and FBC	Mouse IV	Mouse IX	
	Rt: 1.33[a]	Rt: 4.55[b]	
	Lt: 1.34[a]	Lt: 4.78[b]	
	Mouse V	Mouse X	
	Rt: 1.78[a]	Rt: 4.45[b]	
	Lt: 1.62[a]	Lt: 4.58[b]	

Values are ratios to those for age-matched control mice. Each sample was tested in duplicate for three times and results are mean values. Rt, right hemisphere; Lt, left hemisphere.
[a] Remarkable decrease.
[b] Slight decrease of ganglioside GM1 compared with non-treated mice.

was found in the mice of every group. However, at 8 weeks, decrease was detected only in the mouse treated with FBC. Efficacy was still noted at 6 months after injection in FBC-treated mouse. These findings were consistent with those for X-Gal staining (Fig. 1) and β-galactosidase activity (Table 2).

4. Discussion

Two therapeutic methods, HSCT and ERT, are clinically available for LSDs. However, neither is markedly effective in the brain. A number of experiments in animal models have been carried out on the treatment of brain in LSDs. Each revealed some efficacy in the brain, though it was transient and incomplete. Sufficient enzyme expression throughout life is needed in the brain. Thus, permanent engraftment of enzyme-secreting cells in the brain, or permanent expression of an exogenous gene with a vector or as an integrated gene might eliminate the brain involvement in LSDs.

However, the immune responses of host animals are among the most difficult problems to overcome in this respect [31–33]. Although the brain, which is sequestered from systemic immune responses, is thought to exhibit little immune response, elimination of cells expressing a therapeutic transgene occurs in the brain. We speculate that innate inflammatory immune responses are stimulated to kill such cells, not necessarily with the induction of a linked adaptive immune response. When host brain cells express a therapeutic transgene mediated by a viral vector, the host cells themselves will be eliminated, possibly resulting in acceleration of neuronal cell death in neurodegenerative disorders. Transplantation of cells having the same genetic information as the host animals with LSD except for expression of a deficient enzyme protein would thus be a good method of treatment for avoiding the elimination of host neuronal cells and curing diseased host cells.

We performed cell transplantation into the brain of β-galactosidase-deficient mice to study the usefulness of long-term engraftment for supplementation of deficient enzyme protein. To minimize the immune responses in the recipient β-galactosidase knock-out mice, we used cells of mice with the same genetic background as the recipient except for possession of copies of the human β-galactosidase gene.

Initially, in the transplantation experiment, we used FBC from transgenic mice expressing the human β-galactosidase gene. The cells could grow in an environment similar to that of the recipient organ in which they were originally growing. The cells spread into the brains and the cell number increased at least until 4 weeks. They grew very successfully for at least 8 weeks and survived for 6 months or more. However, the number of engrafted cells had decreased significantly at 6 months, while the size of the brain had increased. The decrease in ganglioside GM1 accumulation was also marked until 8 weeks after transplantation. However, at 6 months, this decrease was far less pronounced, with re-accumulation of ganglioside GM1. After the cells were engrafted and the cell number was increased by the cell division in the recipient brain, they were depleted. The mechanism of depletion of transplanted cells involved immunological rejection, although the transplanted cells were very similar genetically and physiologically to the recipient.

Next, we performed a transplantation experiment using MSCs obtained from the bone marrow of the

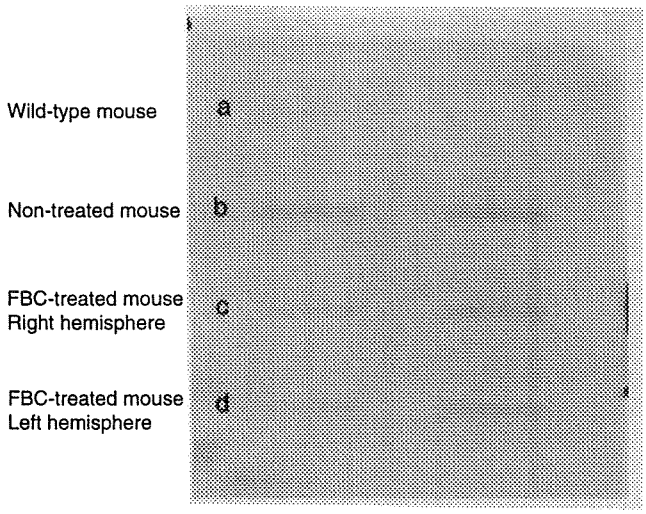

Fig. 2. Immunoblot assay of ganglioside GM1 in brain homogenate at 8 weeks after treatment. Performed in duplicate as shown in two slots for each sample. (a) Wild-type mouse; (b) Non-treated mouse; (c and d) Right and left hemisphere, respectively, of a mouse treated with FBC. The immunoreactivity against ganglioside GM1 antibody in the treated brain (c and d) was less than non-treated brain (d). The accumulated amounts of ganglioside GM1 were calculated in the ratio to the age-matched wild-type mouse (a) from the densitometric quantification signals. These values were shown in Table 3.

same mice expressing the human β-galactosidase gene. MSCs were obtained using the method of plastic adherence. This relatively crude procedure produces a heterogeneous population including multipotential MSCs. These crude cells were used to avoid depletion of potentially important cells and for ease of preparation for clinical application. The cells spread into the brains and the cell number increased similarly to FBC transplantation experiment until 4 weeks. However, decrease in number of engrafted living cells and efficacy in preventing accumulation of ganglioside GM1 were observed in the examination of 8-week-old treated mice.

A number of studies on neural transdifferentiation have been reported [34–37]. Some have reported that neural transdifferentiation of MSCs is induced by cell fusion with host neuronal cells [38–41]. We therefore used mixed FBC and MSC cells to stimulate cell fusion. More engrafted cells were found in the deep areas of the mouse brains treated with mixed cells than in the brains treated with MSC alone. However, no fused cells could be identified. The long-living cells were probably transplanted FBC themselves.

Decrease of ganglioside GM1 was observed even though the increase of the β-galactosidase activity was so small. Similar efficacy was shown previously in our gene therapy experiment [2]. On the other hand, we observed a general depletion of the transplanted cells over time in the BKO mouse brains. The transplanted cells survived in early stage and the number increased by cell division, then, died. This was likely caused by immunological rejection, even though we used fetal brain cells (FBC) from mice with the same genetic background for transplantation. We speculated that immunological reaction occurred because these cells expressed the therapeutic enzyme protein which the host animals did not have. The same has been reported in the transplantation of autogenous cells expressing an exogenous therapeutic gene [33]. The grafted cells were gradually depleted because of immunological rejection by the host animals. To avoid deleterious immune attack and to achieve sufficient long-term efficacy in brain, development of methods to steer the immune response away from cytotoxic responses or to induce tolerance to the products of therapeutic genes is needed [42,43].

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References

[1] Shen JS, Watabe K, Ohashi T, Eto Y. Intraventricular administration of recombinant adenovirus to neonatal twitcher mouse leads to clinicopathological improvements. Gene Ther 2001;8:1081–7.

[2] Takaura N, Yagi T, Maeda M, Nanba E, Oshima A, Suzuki Y, et al. Attenuation of ganglioside GM1 accumulation in the brain of GM1 gangliosidosis mice by neonatal intravenous gene transfer. Gene Ther 2003;10:1487–93.

[3] Kim EY, Hong YB, Lai Z, Cho YH, Brady RO, Jung SC. Long-term expression of the human glucocerebrosidase gene in vivo after transplantation of bone-marrow-derived cells transformed with a lentivirus vector. J Gene Med 2005;7:878–87.

[4] Shen JS, Meng XL, Yokoo T, Sakurai K, Watabe K, Ohashi T, et al. Widespread and highly persistent gene transfer to the CNS by retrovirus vector in utero: implication for gene therapy to Krabbe disease. J Gene Med 2005;7:540–51.

[5] Cachón-González MB, Wang SZ, Lynch A, Ziegler R, Cheng SH, Cox TM. Effective gene therapy in an authentic model of Tay-Sachs-related diseases. Proc Natl Acad Sci USA 2006;103:10373–8.

[6] Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci USA 1999;96:10711–6.

[7] Jin HK, Carter JE, Huntley GW, Schuchman EH. Intracerebral transplantation of mesenchymal stem cells into acid sphingomyelinase-deficient mice delays the onset of neurological abnormalities and extends their life span. J Clin Invest 2002;109:1183–91.

[8] Sakurai K, Iizuka S, Shen JS, Meng XL, Mori T, Umezawa A, et al. Brain transplantation of genetically modified bone marrow stromal cells corrects CNS pathology and cognitive function in MPS VII mice. Gene Ther 2004;11:1475–81.

[9] Givogri MI, Galbiati F, Fasano S, Amadio S, Perani L, Superchi D, et al. Oligodendroglial progenitor cell therapy limits central

neurological deficits in mice with metachromatic leukodystrophy. J Neurosci 2006;26:3109–19.
[10] Kakkis E, McEntee M, Vogler C, Le S, Levy B, Belichenko P, et al. Intrathecal enzyme replacement therapy reduces lysosomal storage in the brain and meninges of the canine model of MPS I. Mol Genet Metab 2004;83:163–74.
[11] Dickson P, McEntee M, Vogler C, Le S, Levy B, Peinovich M, et al. Intrathecal enzyme replacement therapy: successful treatment of brain disease via the cerebrospinal fluid. Mol Genet Metab 2007;91:61–8.
[12] Vogler C, Levy B, Grubb JH, Galvin N, Tan Y, Kakkis E, et al. Overcoming the blood–brain barrier with high-dose enzyme replacement therapy in murine mucopolysaccharidosis VII. Proc Natl Acad Sci USA 2005;102:14777–82.
[13] Matzner U, Herbst E, Hedayati KK, Lüllmann-Rauch R, Wessig C, Schröder S, et al. Enzyme replacement improves nervous system pathology and function in a mouse model for metachromatic leukodystrophy. Hum Mol Genet 2005;14:1139–52.
[14] Blanz J, Stroobants S, Lüllmann-Rauch R, Morelle W, Lüdemann M, D'Hooge R, et al. Reversal of peripheral and central neural storage and ataxia after recombinant enzyme replacement therapy in {alpha}-mannosidosis mice. Hum Mol Genet 2008;17:3437–45.
[15] Grubb JH, Vogler C, Levy B, Galvin N, Tan Y, Sly WS. Chemically modified {beta}-glucuronidase crosses blood–brain barrier and clears neuronal storage in murine mucopolysaccharidosis VII. Proc Natl Acad Sci USA 2008;105:2616–21.
[16] Montaño AM, Oikawa H, Tomatsu S, Nishioka T, Vogler C, Gutierrez MA, et al. Acidic amino acid tag enhances response to enzyme replacement in mucopolysaccharidosis type VII mice. Mol Genet Metab 2008;94:178–89.
[17] Kasperzyk JL, El-Abbadi MM, Hauser EC, D'Azzo A, Platt FM, Seyfried TN. *N*-butyldeoxygalactonojirimycin reduces neonatal brain ganglioside content in a mouse model of GM1 gangliosidosis. J Neurochem 2004;89:645–53.
[18] Lachmann RH, te Vruchte D, Lloyd-Evans E, Reinkensmeier G, Sillence DJ, Fernandez-Guillen L, et al. Treatment with miglustat reverses the lipid-trafficking defect in Niemann-Pick disease type C. Neurobiol Dis 2004;16:654–8.
[19] Cox TM. Substrate reduction therapy for lysosomal storage diseases. Acta Paediatr Suppl. 2005;94:69–75.
[20] Piotrowska E, Jakóbkiewicz-Banecka J, Barańska S, Tylki-Szymańska A, Czartoryska B, Wegrzyn A, et al. Genistein-mediated inhibition of glycosaminoglycan synthesis as a basis for gene expression-targeted isoflavone therapy for mucopolysaccharidoses. Eur J Hum Genet 2006;14:846–52.
[21] Ishii S, Yoshioka H, Mannen K, Kulkarni AB, Fan JQ. Transgenic mouse expressing human mutant alpha-galactosidase A in an endogenous enzyme deficient background: a biochemical animal model for studying active-site specific chaperone therapy for Fabry disease. Biochim Biophys Acta 2004;1690:250–7.
[22] Matsuda J, Suzuki O, Oshima A, Yamamoto Y, Noguchi A, Takimoto K, et al. Chemical chaperone therapy for brain pathology in G(M1)-gangliosidosis. Proc Natl Acad Sci USA 2003;100:15912–7.
[23] Matsuda J, Suzuki O, Oshima A, Ogura A, Noguchi Y, Yamamoto Y, et al. Beta-galactosidase-deficient mouse as an animal model for GM1-gangliosidosis. Glycoconj J 1997;14:729–36.
[24] Yamamoto Y, Nagase Y, Noguchi A, Mochida K, Nakahira M, Takano K, et al. Generation and characterization of the β-galactosidase knockout mouse having the normal human β-galactosidase gene as a transgene (in Japanese). Proc Jap Soc of Animal Models for Hum Dis (Nippon Shikkan Model Gakkai Kiroku) 2001;17:20–2.
[25] Meirelles Lda S, Nardi NB. Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. Br J Haematol 2003;123:702–11.
[26] Meberg PJ, Miller MW. Culturing hippocampal and cortical neurons. Methods Cell Biol 2003;71:111–27.
[27] Suzuki K. Enzymatic diagnosis of sphingolipidosis. Methods Enzymol 1987;138:727–62.
[28] Bradford MM. A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:255–60.
[29] Michikawa M, Gong JS, Fan QW, Sawamura N, Yanagisawa K. A novel action of alzheimer's amyloid beta-protein (Abeta): oligomeric Abeta promotes lipid release. J Neurosci 2001;21:7226–35.
[30] Suzuki Y, Ichinomiya S, Kurosawa M, Ohkubo M, Watanabe H, Iwasaki H, et al. Chemical chaperone therapy: clinical effect in murine G(M1)-gangliosidosis. Ann Neurol 2007;62:671–5.
[31] Barker RA, Widner H. Immune problems in central nervous system cell therapy. NeuroRx 2004;1:472–81.
[32] Abordo-Adesida E, Follenzi A, Barcia C, Sciascia S, Castro MG, Naldini L, et al. Stability of lentiviral vector-mediated transgene expression in the brain in the presence of systemic antivector immune responses. Hum Gene Ther 2005;16:741–51.
[33] Lowenstein PR, Kroeger K, Castro MG. Immunology of neurological gene therapy: how T cells modulate viral vector-mediated therapeutic transgene expression through immunological synapses. Neurotherapeutics 2007;4:715–24.
[34] Weimann JM, Charlton CA, Brazelton TR, Hackman RC, Blau HM. Contribution of transplanted bone marrow cells to Purkinje neurons in human adult brains. Proc Natl Acad Sci USA 2003;100:2088–93.
[35] Abouelfetouh A, Kondoh T, Ehara K, Kohmura E. Morphological differentiation of bone marrow stromal cells into neuron-like cells after co-culture with hippocampal slice. Brain Res 2004;1029:114–9.
[36] Wislet-Gendebien S, Hans G, Leprince P, Rigo JM, Moonen G, Rogister B. Plasticity of cultured mesenchymal stem cells: switch from nestin-positive to excitable neuron-like phenotype. Stem cells 2005;23:392–402.
[37] Deng J, Petersen BE, Steindler DA, Jorgensen ML, Laywell ED. Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation. Stem cells 2006;24:105410–64.
[38] Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 2002;416:542–5.
[39] Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, et al. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 2003;425:968–73.
[40] Kozorovitskiy Y, Gould E. Stem cell fusion in the brain. Nat Cell Biol 2003;5:952–4.
[41] Bae JS, Furuya S, Shinoda Y, Endo S, Schuchman EH, Hirabayashi Y, et al. Neurodegeneration augments the ability of bone marrow-derived mesenchymal stem cells to fuse with Purkinje neurons in Niemann-Pick type C mice. Hum Gene Ther 2005;16:1006–11.
[42] Tomatsu S, Gutierrez M, Nishioka T, Yamada M, Yamada M, Tosaka Y, et al. Development of MPS IVA mouse (Galnstm(hC79S. mC76S)slu) tolerant to human *N*-acetylgalactosamine-6-sulfate sulfatase. Hum Mol Genet 2005;14:3321–5.
[43] Matzner U, Matthes F, Herbst E, Lüllmann-Rauch R, Callaerts-Vegh Z, D'Hooge R, et al. Induction of tolerance to human arylsulfatase A in a mouse model of metachromatic leukodystrophy. Mol Med 2007;13:471–9.

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ORIGINAL ARTICLE

Mucolipidosis II and III alpha/beta: mutation analysis of 40 Japanese patients showed genotype–phenotype correlation

Takanobu Otomo[1], Takeshi Muramatsu[1], Tohru Yorifuji[2], Torayuki Okuyama[3], Hiroki Nakabayashi[4], Toshiyuki Fukao[5], Toshihiro Ohura[6], Makoto Yoshino[7], Akemi Tanaka[8], Nobuhiko Okamoto[9], Koji Inui[10], Keiichi Ozono[1] and Norio Sakai[1]

Mucolipidosis (ML) II alpha/beta and III alpha/beta are autosomal recessive diseases caused by a deficiency of α and/or β subunits of the enzyme *N*-acetylglucosamine-1-phosphotransferase, which is encoded by the *GNPTAB* gene. We analyzed the *GNPTAB* gene in 25 ML II and 15 ML III Japanese patients. In most ML II patients, the clinical conditions 'stand alone', 'walk without support' and 'speak single words' were impaired; however, the frequency of 'heart murmur', 'inguinal hernia' and 'hepatomegaly and/or splenomegaly' did not differ between ML II and III patients. We detected mutations in *GNPTAB* in 73 of 80 alleles. Fourteen new mutations were c.914_915insA, c.2089_2090insC, c.2427delC, c.2544delA, c.2693delA, c.3310delG, c.3388_3389insC+c.3392C>T, c.3428_3429insA, c.3741_3744delAGAA, p.R334L, p.F374L, p.H956Y, p.N1153S and duplication of exon 2. Previously reported mutations were p.Q104X, p.W894X, p.R1189X and c.2715+1G>A causing skipping of exon 13. Homozygotes or compound heterozygotes of nonsense and frameshift mutations contributed to the severe phenotype. p.F374L, p.N1153S and splicing mutations contributed to the attenuated phenotype, although coupled with nonsense mutation. These results show the effective molecular diagnosis of ML II and III and also provide phenotypic prediction. This is the first and comprehensive report of molecular analysis for ML patients of Japanese origin.
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Keywords: genotype–phenotype correlation; *GNPTAB*; *GNPTG*; I-cell disease; Japanese; mucolipidosis II alpha/beta; mucolipidosis III alpha/beta; mutation analysis

INTRODUCTION

Mucolipidosis (ML) is clinically characterized by developmental delay and dysostosis multiplex, which is partially overlapped with mucopolysaccharidoses. ML II and III are autosomal recessive diseases caused by reduced enzyme activity of *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) (EC 2.7.8.17). Targeting of newly synthesized lysosomal enzymes to lysosomes is mediated mainly by mannose-6-phosphate receptor, which recognizes the phosphate at the end of the sugar chain on lysosomal enzymes. The recognition marker is synthesized in a two-step reaction and GlcNAc-phosphotransferase acts in the first step. In patients, targeting of many lysosomal enzymes to the lysosome is impaired and levels of overflowed lysosomal enzymes are elevated in the serum and body fluids.[1]

Structural analysis of bovine GlcNAc-phosphotransferase shows that this enzyme is an $\alpha_2\beta_2\gamma_2$ hexameric peptide complex.[2] In 2000, Raas-Rothschild *et al.*[3] reported that the γ subunit is encoded by the *GNPTG* gene and contributes to the pathology of ML III gamma, formerly described as ML IIIC.[4] Recent cloning of cDNAs for α/β subunits showed that it is encoded by a single gene *GNPTAB*.[5] *GNPTAB* is located at chromosome 12q23.3, contains 21 exons and codes 1256 amino acids. The α–β boundary is located within exon 14, but the detailed mechanism of processing the precursor into α and β subunits is not clear.[6] A series of genetic-complementation studies have shown heterogeneity in ML III and the genetic relationship between ML II and III.[7–9] Mutations in *GNPTAB* cause both the severe type of ML (ML II alpha/beta, ML II, I-cell disease (MIM 252500)) and the attenuated type of ML (ML III alpha/beta, ML IIIA, Pseudo-Hurler polydystrophy (MIM 252600)).[10–12] Mutations in *GNPTG* cause the attenuated type of ML (ML III gamma, ML IIIC, ML III variant (MIM 252605)).

[1]Department of Pediatrics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan; [2]Department of Pediatrics, Kyoto University Hospital, Kyoto, Japan; [3]Department of Clinical Genetics and Molecular Medicine, National Center for Child Health and Development, Tokyo, Japan; [4]Department of Pediatrics, Surugadai Nihon University Hospital, Tokyo, Japan; [5]Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan; [6]Department of Pediatrics, Sendai City Hospital, Sendai, Japan; [7]Department of Pediatrics and Child Health, Kurume University School of Medicine, Kurume, Japan; [8]Department of Pediatrics, Osaka City University School of Medicine, Osaka, Japan; [9]Department of Planning and Research, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan and [10]Inui Children's Clinic, Itami, Japan
Correspondence: Dr N Sakai, Department of Pediatrics (D-5), Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan.
E-mail: norio@ped.med.osaka-u.ac.jp
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We analyzed the *GNPTAB* gene in 40 Japanese ML II and III patients who had been diagnosed clinically or biochemically. Because the previously recorded clinical information was ambiguous, we reviewed the clinical records of these patients. In this article, we show the results of mutation analysis of the *GNPTAB* gene in 40 Japanese ML II alpha/beta and III alpha/beta patients and the comparison of the obtained genotype and phenotypes.

MATERIALS AND METHODS

Patients

The cases were Japanese ML II and III patients diagnosed after the 1970s to date. The diagnosis of ML was based on clinical manifestations and lysosomal enzyme activities in serum, lymphocyte and skin fibroblasts. The number of samples was 40, including 25 samples of ML II and 15 samples of ML III. In this study, all patients were probands and no siblings were included. New patients were informed about the gene test by counselors in each institute and consented. All methods in this study were approved by the ethics committee of Osaka University Graduate School of Medicine.

Clinical information

The clinical information of each patient followed in our outpatients' clinic was reviewed from the medical records at our institute. To obtain clinical information about patients followed in other institutes, we sent questionnaires to outpatients' doctors or patients' families. Many medical records had been discarded because of the long period since their death or the end of follow-up. In some cases (cases 4, 12, 13, 14 and 34), only information, such as 'ML II patient' or 'I-cell disease', was available.

Cell culture

Skin fibroblasts of patients were stored in liquid nitrogen at −196 °C. Half of each thawed sample was subjected to re-culture with Dulbecco's modified Eagle's medium (GIBCO; Grand Island, NY, USA) with 10% fetal bovine serum and anti-biotic-anti-mycotic (GIBCO). The other half of each frozen sample was directly subjected to RNA and DNA extraction.

Total RNA and genomic DNA extraction

We extracted total RNA and genomic DNA using a standard extraction kit (Isogen; Nippongene, Tokyo, Japan) from patients' peripheral blood leukocytes and/or cultured skin fibroblasts.

Reverse transcription

The obtained total RNA was subjected to reverse transcription to construct cDNA. Synthesis of cDNA was performed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Amplification of cDNA and genomic DNA

A cDNA fragment covering the whole coding region of *GNPTAB* was amplified by PCR in two fragments (first PCR) and each fragment was secondarily amplified in two fragments (second PCR). In other words, whole cDNA was amplified in four overlapping fragments. Six pairs of primer sequences for amplification of cDNA are listed in Supplementary 1. For genomic DNA amplification of each exon of *GNPTAB* by PCR, we used genomic primers described earlier by Kudo *et al.*[10] (primer ID; 1088, 1076, 1077, 1078, 1082, 1118, 1119, 1085, 1086, 1089, 1107, 1092, 1109, 1120, 1121, 1122, 1123, 1163, 1164, 1129, 1134, 1135, 1136, 1215, 1216, 1139, 1140, 1141, 1142, 1219 and 1259) and other newly designed primers (Supplementary 1). For the amplification of each exon of *GNPTG*, we used primers described earlier by Raas-Rothschild *et al.*[3] and other newly designed primers (Supplementary 1). PCR reactions were conducted in 20 µl reaction volumes with rTaq DNA polymerase (Takara Bio Inc., Otsu, Japan). The basic thermal profile consisted of initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 56 °C for 2 min and 72 °C for 2 min, with a final extension at 72 °C for 7 min. Annealing temperature was modified within 55 and 60 °C. Three microliters of each PCR mixture were run on agarose gel to ensure that only the specific product was amplified.

DNA sequencing

PCR products were purified using a standard kit (SUPREC-02; Takara Bio Inc.) to remove unnecessary primers and finally dissolved in pure water at a concentration of approximately 2–10 ng µl^{-1} water as a sequencing reaction template. DNA sequencing was carried out with the same primers as mentioned above (Supplementary 1) using the BigDye Terminator V1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the Applied Biosystems 3100 genetic analyzer according to the manufacturer's instructions. First, DNA sequencing was performed for cDNAs of *GNPTAB* followed by the confirmation by genomic DNA sequencing. We also analyzed the genomic DNA sequence of *GNPTG* in seven patients in whom only one mutation in *GNPTAB* was detected.

Screening for p.F374L and p.R1189X mutation by restriction fragment length polymorphism

DNA fragments containing the mutation site were amplified by PCR with primer 343/1109 (Supplementary 1) (Kudo *et al.*[10]) and 230/232 (Supplementary 1) and digested by restriction enzyme *Mbo* II or *Taq* I, respectively. PCR fragments containing p.F374L were recognized by *Mbo* II and digested from 367 to 33+16+97+221 bp. The same fragments derived from the normal allele were digested from 367 to 33+16+318 bp. With regard to PCR fragments containing p.R1189X, *Taq* I recognizes only the normal sequence and digests the fragment of 293 bp length into 205+88 bp fragments. If mutations were found by this method, DNA sequences were confirmed by genomic DNA sequencing by the same method as described above.

RESULTS

Mutations in ML II alpha/beta and III alpha/beta patients

We detected 73 mutant alleles of 80 alleles in 40 Japanese patients (Table 1). Bold letters indicate new mutations detected in this study. These include 14 new mutations and four previously detected mutations. All four known mutations were reported by a Korean group[11] and one, p.R1189X, was also reported by an Israeli group in a patient of Irish/Scottish origin.[13] The most frequent mutation was the nonsense mutation p.R1189X (c.3565C>T) and its allele frequency was 33/80 (=41.25%) in the analyzed alleles of all ML II and III patients. In 90 healthy individuals, this mutation was not found by the restriction fragment length polymorphism method described above. p.F374L was found in 1 of 25 ML II patients and 7 of 15 ML III patients, and total allele frequency was 8/80 (=10.00%). Duplication of exon 2 was found in 1 of 25 ML II patients and 5 of 15 ML III patients, and total allele frequency was 6/80 (=7.50%). No mutation was found in *GNPTG* in seven patients in whom only one mutation was detected in *GNPTAB*.

Analysis of the duplication of exon 2

First, we found mutant alleles with the structure of duplicating exon 2 in cDNA analysis (Figure 1c). We constructed forward and reverse primers within exon 2 (primer 336/335) and performed long PCR with an XL PCR Kit (Applied Biosystems). The basic thermal profile consisted of initial denaturation at 94 °C for 1 min, followed by 35 cycles at 94 °C for 15 s, 62 °C for 12 min, with a final extension at 72 °C for 15 min. None was amplified with normal alleles by this method because each primer runs the other way. With a mutant allele, a specific fragment was amplified and the size of the fragment was estimated as approximately 15 kbp by agarose gel electrophoresis (Figure 1d). Sequencing analysis of this fragment was performed by primer walking and the junction between introns 1 and 2 was confirmed (Figure 1b); however, we could not detect any rearrangement between exons 1 and 2 by primer walking (data not shown).

Table 1 Summary of all mutations in *GNPTAB* identified in this study

Case	Position	DNA	Protein
ML II alpha/beta			
1	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
2	**Exon 13**	**c.2089_2090insC**	**p.L697fs**
	Exon 19	c.3565C>T	p.R1189X
3	**Exon 8**	**c.914_915insA**	**p.D305fs**
	Exon 19	c.3565C>T	p.R1189X
4	Exon 19	c.3565C>T	p.R1189X
	ND	ND	ND
5	Exon 13	c.2681G>A	p.W894X
	Exon 19	c.3565C>T	p.R1189X
6	**Exon 10**	**c.1120T>C**	**p.F374L**
	ND	ND	ND
7	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
8	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
9	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
10	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
11	**Exon 13**	**c.2427delC**	**p.L810fs**
	Exon 19	c.3565C>T	p.R1189X
12	**Exon 18**	**c.3388_3389insC+c.3392C>T**	**p.V1130fs**
	ND	ND	ND
13	**Exon 9**	**c.1001G>T**	**p.R334L**
	Exon 19	c.3565C>T	p.R1189X
14	**Exon 2**	**Duplication exon 2**	**Frameshift**
	Exon 13	**c.2544delA**	**p.K848fs**
15	Exon 3	c.310C>T	p.Q104X
	Exon 19	c.3565C>T	p.R1189X
16	Exon 3	c.310C>T	p.Q104X
	Exon 3	c.310C>T	p.Q104X
17	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
18	**Exon 17**	**c.3310delG**	**p.A1104fs**
	Exon 18	**c.3428_3429insA**	**p.N1143fs**
19	Exon 19	c.3565C>T	p.R1189X
	ND	ND	ND
20	**Exon 21**	**c.3741_3744delAGAA**	**p.E1248fs**
	ND	ND	ND
21	**Exon 13**	**c.2544delA**	**p.K848fs**
	Exon 13	**c.2544delA**	**p.K848fs**
22	Exon 3	c.310C>T	p.Q104X
	Exon 19	c.3565C>T	p.R1189X
23	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
24	Exon 19	c.3565C>T	p.R1189X
	ND	ND	ND
25	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
ML III alpha/beta			
26	Intron 13	c.2715+1G>A	(skip Exon 13)
	Exon 19	c.3565C>T	p.R1189X
27	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 19	c.3565C>T	p.R1189X
28	**Exon 2**	**Duplication exon 2**	**Frameshift**
	Exon 13	**c.2089_2090insC**	**p.L697fs**
29	**Exon 2**	**Duplication exon 2**	**Frameshift**
	Exon 13	**c.2693delA**	**p.K898fs**
30	**Exon 2**	**Duplication exon 2**	**Frameshift**
	ND	ND	ND
31	**Exon 2**	**Duplication exon 2**	**Frameshift**
	Exon 13	**c.2544delA**	**p.K848fs**
32	**Exon 2**	**Duplication exon 2**	**Frameshift**
	Exon 10	**c.1120T>C**	**p.F374L**
33	**Exon 19**	**c.3458A>G**	**p.N1153S**
	Exon 19	c.3565C>T	p.R1189X
34	Intron 13	c.2715+1G>A	(skip Exon 13)
	Exon 14	**c.2866C>T**	**p.H956Y**
35	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 13	c.2681G>A	p.W894X
36	Intron 1	c.2715+1G>A	(skip Exon 13)
	Exon 14	**c.2866C>T**	**p.H956Y**
37	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 19	c.3565C>T	p.R1189X
38	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 19	c.3565C>T	p.R1189X
39	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 19	c.3565C>T	p.R1189X
40	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 19	c.3565C>T	p.R1189X

Abbreviations: ML, mucolipidosis; ND, not detectable.
Bold letters indicate novel mutations detected in this study. Numbering of nucleotides starts with +1 at the first nucleotide of the initiation codon and numbering of amino acid starts with the first methionine encoded by the ATG. Descriptions about cDNA mutation and mutant protein mainly conform to the 'Nomenclature for the description of sequence variations' by Human Genome Variation Society (http://www.hgvs.org/mutnomen/).

Polymorphisms in *GNPTAB*

The 14 polymorphisms in *GNPTAB* found in this study are listed in Table 2. Ten polymorphisms were found within the intron and four within the coding region that does not change the amino-acid residue. Not all patients were tested because these polymorphisms were found incidentally in the process of sequencing analysis of mutations. Twelve polymorphisms have been registered in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). Two polymorphisms have not been reported previously.

Clinical phenotypes and genotypes

A summary of the clinical images and types of mutations of patients is shown in Table 3. We chose simple clinical manifestations, such as standing or walking, speaking, heart murmur, inguinal hernia and hepatosplenomegaly, which could be easily retrieved from old medical records or the memory of the doctors who followed them. Only the ML type was recorded in some cases but we could not obtain further information. The frequency of each clinical manifestation in the two groups is shown in Table 4.

In 33 patients, mutation was detected in both alleles. We arranged all cases into eight groups based on the type of mutation, respectively (Table 3).

Nonsense mutation contains p.Q104X, p.W894X and p.R1189X. Frameshift contains a type of mutation caused by the insertion or deletion of 1 or 4 bases, including c.914_915insA, c.2089_2090insC, c.2427delC, c.2544delA, c.2693delA, c.3310delG, c.3388_3389insC +c.3392C>T, c.3428_3429insA and c.3741_3744delAGAA. Each missense mutation, p.R334L, p.F374L, p.H956Y and p.N1153S, is

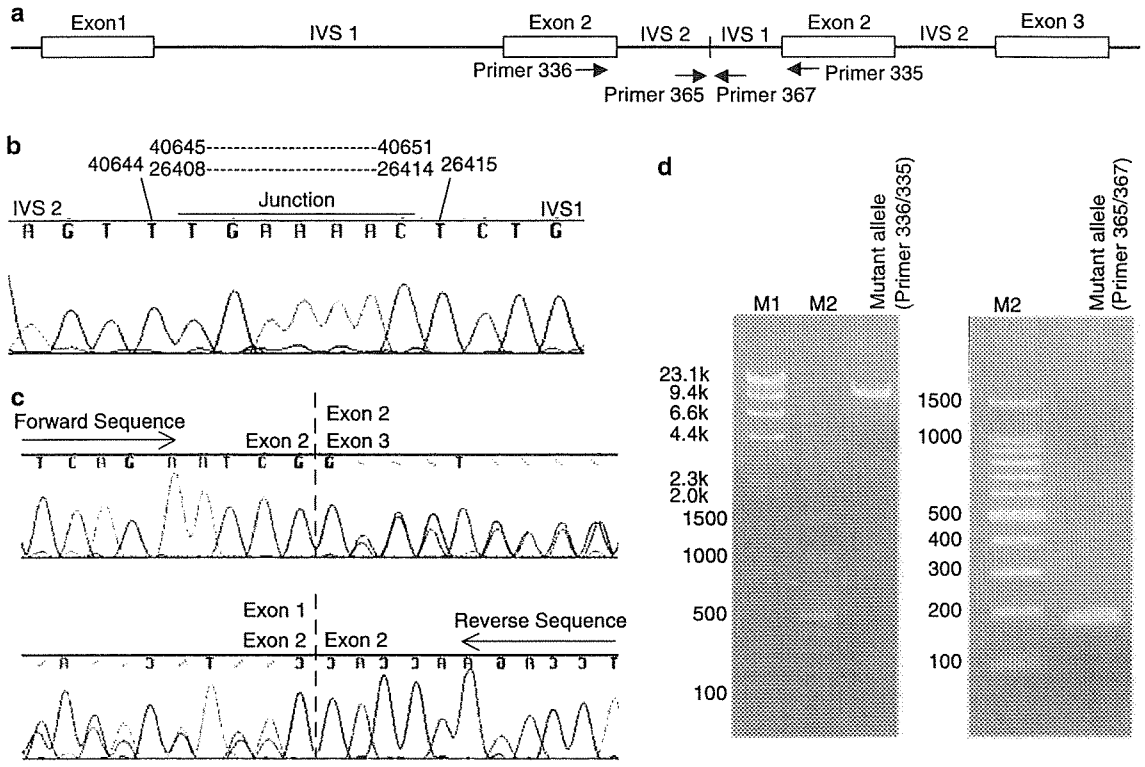

Figure 1 Summary of the duplication exon 2. (**a**) Overview of rearranged mutant allele. (**b**) Genomic sequence around the junction. XL PCR product of primer set 336/335 was used as a sequence template. Sequencing analysis of mutant allele showed that IVS 2 jumped and connected to IVS 1 through 7 overlapping bases. The number above indicates the position in reference sequence NT_019546.15. (**c**) Sequence analysis of cDNA. Forward sequencing showed overlapped signal of exons 2 and 3 next to the end of exon 2. Reverse sequencing also showed overlapped signal of exons 1 and 2 next to the end of exon 2. (**d**) Agarose gel electrophoresis of PCR products amplified by primer set 336/335 and 365/367. These primers were designed to run the other way, respectively, and only the mutant allele was amplified. Left figure indicates that the amplified fragment by primer set 336/335 was estimated to be about 15 kbp. We then sequenced the fragment by primer walking. The junction was found and primers were designed near the junction. Right figure indicates the PCR fragment amplified by primer set 365/367. Estimated fragment size was 177 bp. M1 is a ladder of λ/*Hind* III marker and M2 is a ladder of 100 bp marker (Takara Bio Inc.).

Table 2 Polymorphisms in *GNPTAB*

Position[a]	*Location in GNPTAB*	*Genomic DNA polymorphism*[b]	*cDNA*[c]
25706627	Exon 1	G>A	c.18G>A (synonymous)
25666041	Intron 2	G>T	
25665434_36	Intron 3	ATA>A (del2bases)[d]	
25665015	Intron 3	A>G	
25664898	Intron 3	T>A	
25664420_24	Intron 4	TGTGT>TGT (del2bases)	
25664372	Intron 4	C>T	
25662419	Intron 4	G>A	
25641086	Exon 13	C>G[d]	c.1800C>G (synonymous)
25640954	Exon 13	A>G	c.1932A>G (synonymous)
25640405	Exon 13	C>T	c.2481C>T (synonymous)
25637091	Intron 15	T>C	
25633304	Intron 17	T>C	
25633069	Intron 18	C>G	

[a]Position in the reference sequence NT_019546.15.
[b]Nucleotide changes from the reference sequence NT_019546.15.
[c]Numbering of nucleotides starts with +1 at the first nucleotide of the initiation codon.
[d]New polymorphisms detected in this study.

enumerated individually. Duplication of exon 2 and skipping of exon 13 are also enumerated individually, although they finally cause frameshift. Homozygotes or compound heterozygotes of the mutation are indicated by a closed circle and heterozygotes by an open circle.

DISCUSSION

In this study, several frequent mutations were detected. Nonsense mutation c.3565C>T (p.R1189X) was especially frequent in our study and its allele frequency was 41.25%. Eight of 25 ML II patients have this mutation homozygously. According to previous reports, this mutation was only found in two Korean cases[11] and in one case of Irish/Scottish origin.[13] This mutation seems to be common in eastern Asia, including Japan. It is speculated that it does not occur in a hot spot but by a founder effect, although experimental confirmation is difficult because of the unavailability of samples from the patients' parents. The secondary frequent mutation is a missense mutation c.1120C>T (p.F374L) found in eight alleles compounded heterozygously with another mutation in this study. Duplication of exon 2 was found in six alleles and this mutation occurred at the genomic level of rearrangement. Sequencing analysis of the fragment from the tail of exon 2 to the head of exon 2 showed the junction between introns 1

Table 3 Summary of clinical images and mutation types in Japanese mucolipidosis (ML) II and III alpha/beta patients

	ML II alpha/beta																				*ML III alpha/beta*																				
Case number	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*		
Age (years) (×age at death)	*9*	*(3)×*	*(1)×*			*(6)×*		*(6)×*	*(2)×*							*(1)×*		*7*		*(13)×*		*(2)×*		*(6)×*	*2*	*(20)×*	*8*		*(17)×*	*28*			*32*		*(20)×*		*24*	*3*	*15*	*37*		
Stand alone	–	–			+				–	–	–					–	–	–	–	–	–				–	+	+	+	+	+	+		+		+	+	+	+	+	+		
Walk without support	–	–			–				–	–	–					–	–	–	–	–	–				–	+	+	+	+	+	+		+		–	+	+	+	+	+		
Speak single words	–								–	–	–					–	–	–	–	–	–				–	+	+	+	+	+	+		+		–	+	+	+	+	+		
Heart murmur	+	+	+			–			+	+	+					+	+	+	+	+	+	+	+		–	–	+	+	+	+	+		+		+	+	–	–	+		
Inguinal hernia	+		+			+	–		+	+	+					–	–	–	+	+	+				+	–	+	–	+	+					–	+	–	–	+		
Hepatomegaly and/or splenomegaly	+	+	+		+	+	+	+	+	+	+					+	+	+	–	+	+	+	+		–	–	+	+	+	–	+		–		+	+	–	–	+		
Nonsense																																										
Frameshift																																										
Missense p.R334L																																										
Missense p.F374L																																										
Missense p.H956Y																																										
Missense p.N1153S																																										
Duplication of exon 2																																										
Skipping of exon 13																																										

Table 4 Clinical manifestations and mucolipidosis (ML) types

Clinical manifestations	*ML II*	*ML III*
Stand alone	1/13	13/13
Walk without support	0/15	12/13
Speak single words	1/13	12/13
Heart murmur	15/17	12/14
Inguinal hernia	10/15	5/9
Hepatomegaly and/or splenomegaly	17/20	6/12

and 2; however, there was no junction between exons 1 and 2 by primer walking. We suggest that this rearrangement is not derived from insertion of a region containing exon 2 but from the recombination between introns 1 and 2 (Figure 1a). Skipping of exon 13 is also frequent and is caused by the intronic one-base substitution c.2715+1G>A mutation. This mutation is discussed in detail in the previous article.[11]

We examined the founder effects of several mutations based on the result of polymorphisms. We show the genotype frequencies of several polymorphisms in patients with/without p.F374L and c.2715+1G>A (Table 5). Reference frequencies are data from HapMap-JPT (Japanese in Tokyo, Japan) in the International HapMap project. Our results indicate that six single-nucleotide polymorphism (SNPs) (rs10778150, rs2108694, rs6539012, rs10778148, rs759935 and rs376475) are coincident with mutation p.F374L and the SNP (rs3751249) with c.2715+1G>A in Japanese ML patients. We suggest that high correspondence between the two mutations and SNPs is most likely explained by a founder effect. With regard to the other mutations, there seemed to be no relationship between the mutations and polymorphisms.

Traditionally, ML patients have been classified into severe type (as type II) and attenuated type (as type III). We examined genotype–phenotype correlation with the obtained mutational information and patient diagnosis, namely ML type. Patients with a combination of homozygotes or compound heterozygotes within nonsense mutation, p.Q104X, p.W894X and p.R1189X, showed clinically severe phenotypes. Frameshift mutations caused by insertion or deletion of 1 or 4 bases (c.914_915insA, c.2089_2090insC, c.2427delC, c.2544delA, c.2693delA, c.3310delG, c.3388_3389insC+c.3392C>T, c.3428_3429 insA, c.3741_3744delAGAA) also contribute to the severe phenotype.

On the other hand, p.F374L is considered to contribute to relatively mild clinical manifestations, except for case 6, which was reported in detail by Kojima *et al.*[14] and also by Okada *et al.*[15] (case 9 in the article). He could stand alone but could not walk and was classified into ML II. We think that this was not a typical ML II case and showed a relatively attenuated phenotype. The other cases of p.F374L, if combined with nonsense or frameshift mutation, clinically showed ML III.

Duplication of exon 2 was found in attenuated cases, except for case 14; however, we cannot discuss case 14 further because limited information was available. We consider that duplication of exon 2 contributes to the attenuated phenotype. c.2715+1G>A causes skipping of exon 13.[11] Case 26 has p.R1189X and c.2715+1G>A and shows an attenuated clinical phenotype. We consider that c.2715+1G>A also contributes to the attenuated phenotype.

Exon 2 contains 86 base sequences and exon 13 contains 1103 base sequences. The above two mutations consequently lead to frameshift. The Korean group reported skipping of exon 13 only in ML III

Table 5 Genotype frequencies of mutations and polymorphisms

Position[a] *(dbSNP id)*	*Population*	*Genotype frequency*						n
	HapMap-JPT	T/T	0	T/A	0.111	A/A	**0.889**	
25664898 (rs10778150)	Without p.F374L		0		0		**1.000**	8
	p.F374L hetero		0		**1.000**		0	2
	HapMap-JPT	C/C	0	C/T	0.119	T/T	**0.881**	
25664372 (rs2108694)	Without p.F374L		0		0		**1.000**	6
	p.F374L hetero		0		**1.000**		0	5
	HapMap-JPT	G/G	0.023	G/A	0.114	A/A	**0.864**	
25662419 (rs6539012)	Without p.F374L		0		0.143		**0.857**	14
	p.F374L hetero		0		**1.000**		0	6
	HapMap-JPT	A/A	0	A/G	0.244	G/G	**0.756**	
25640954 (rs10778148)	Without p.F374L		0		0.133		**0.867**	15
	p.F374L hetero		0		**1.000**		0	6
	HapMap-JPT	T/T	0	T/C	0.244	C/C	**0.756**	
25637091 (rs759935)	Without p.F374L		0		0		**1.000**	10
	p.F374L hetero		0		**1.000**		0	5
	HapMap-JPT	C/C	0	C/G	0.250	G/G	**0.750**	
25633069 (rs3736475)	Without p.F374L		0		0.143		**0.857**	14
	p.F374L hetero		0		**1.000**		0	4
	HapMap-JPT	C/C	**0.977**	C/T	0.023	T/T	0	
25640405 (rs3751249)	Without c.2715+1G>A		**1.000**		0		0	18
	c.2715+1G>A hetero		0		**1.000**		0	3

'*n*' means the number of patients tested for single-nucleotide polymorphisms.
Bold letters indicate major genotypes within each row.
[a]Position in the reference sequence NT_019546.15.

patients. Kudo *et al.* reported a case in which splicing mutation caused an attenuated phenotype with highly suppressed enzyme activity and suggested that splicing was especially disrupted only in these fibroblasts. Our results are consistent, although the detailed mechanisms remain unclear.

Case 33 has compound heterozygosity of p.R1189X and p.N1153S. This case shows one of the most attenuated phenotypes in this study; she could speak and walk when she was 1 year old, without hepatosplenomegaly, and she is now 32 years old and working in a workshop. p.N1153S was found in only one allele; however, it is suggested that this mutation contributes to the attenuated phenotype.

In this study, clinical severity proved to be well correlated with mutational severity. Our result supports the previous report by Bargal *et al.*[13] On the basis of information about known mutations, a patient's clinical phenotype can be estimated. Even if new mutations are found, some clinical phenotypes can be predicted by the type of mutation.

We investigated the correlation between clinical manifestations and diagnosed ML types (Table 4). 'Stand alone', 'walk without support' and 'speak single words' are considered to well correlate to the ML type. In other words, a patient who cannot stand, walk or speak has a severe phenotype and life expectancy is estimated to be limited, generally called 'ML II'. Clinical manifestations, such as 'heart murmur', 'inguinal hernia' and 'hepatomegaly and/or splenomegaly', were found in both ML II and III. Unfortunately, other various clinical manifestations, such as 'age at onset', 'bone deformity' and 'neurological findings', could not be examined because of poor information about many cases in this study. If a large-scale prospective investigation about the natural history of ML is carried out in the future, these problems may be solved.

Mucolipidosis II alpha/beta and III alpha/beta are caused by the same gene, *GNPTAB*, and the clinical phenotype shows a continuum from mild to severe. In both ML II and III, life expectancy is becoming longer owing to improved supportive therapy and care. Previously classified ML II patients can now live for more than 10 years; however, there are undoubtedly differences between typical ML II and III. It is difficult to predict the clinical phenotype from only enzymatic or biochemical characteristics.[15] From a clinical standpoint, early prediction of prognosis is necessary. We could clearly classify the former ML types from selected clinical manifestations and these ML types correlated with genotypes. This is useful for predicting prognosis to analyze mutations for treatment, including hematopoietic stem cell transplantation, especially in attenuated cases diagnosed in the early stage by molecular analysis.[16]

According to the recent report,[17] 23 different mutations have been reported in the *GNPTAB* gene causing ML II and III alpha/beta. We detected 14 new mutations in the Japanese population. Three of four other already known mutations detected this time were reported only in Korean patient. This indicates that mutations found in Asian people are restricted to the Asian region and seem not to be derived from hot spots.

This is the first and comprehensive report of molecular analysis for Japanese patients of ML. Our data showed genotype–phenotype correlations in Japanese ML II and III (alpha/beta) in particular

mutations. This result provides effective molecular diagnosis and phenotypic prediction of ML II and III (alpha/beta). Additionally, we showed that clinical severity and life expectancy are also predicted by particular clinical manifestations, including standing alone, walking without support and speaking single words.

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1 Kornfeld, S. & Sly, W. S. I-cell disease and Pseudo-Hurler Polydystrophy: Disorders of lysosomal enzyme phosphorylation and localization. In *The Metabolic and Molecular Bases of Inherited Disease* (eds Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D.) Ch. 138, 3469–3482 (McGraw-Hill, New York, 2001).
2 Bao, M., Booth, J. L., Elmendorf, B. J. & Canfield, W. M. Bovine UDP-*N*-acetylglucosamine:lysosomal-enzyme *N*-acetylglucosamine-1-phosphotransferase. I. Purification and subunit structure. *J. Biol. Chem.* **271**, 31437–31445 (1996).
3 Raas-Rothschild, A., Cormier-Daire, V., Bao, M., Genin, E., Salomon, R., Brewer, K. *et al.* Molecular basis of variant pseudo-hurler polydystrophy (mucolipidosis IIIC). *J. Clin. Invest.* **105**, 673–681 (2000).
4 Cathey, S. S., Kudo, M., Tiede, S., Raas-Rothschild, A., Braulke, T., Beck, M. *et al.* Molecular order in mucolipidosis II and III nomenclature. *Am. J. Med. Genet. A* **146A**, 512–513 (2008).
5 Kudo, M., Bao, M., D'Souza, A., Ying, F., Pan, H., Roe, B. A. *et al.* The alpha- and beta-subunits of the human UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase [corrected] are encoded by a single cDNA. *J. Biol. Chem.* **280**, 36141–36149 (2005).
6 Kudo, M. & Canfield, W. M. Structural requirements for efficient processing and activation of recombinant human UDP-*N*-acetylglucosamine:lysosomal-enzyme-*N*-acetylglucosamine-1-phosphotransferase. *J. Biol. Chem.* **281**, 11761–11768 (2006).
7 Honey, N. K., Mueller, O. T., Little, L. E., Miller, A. L. & Shows, T. B. Mucolipidosis III is genetically heterogeneous. *Proc. Natl. Acad. Sci. USA* **79**, 7420–7424 (1982).
8 Mueller, O. T., Honey, N. K., Little, L. E., Miller, A. L. & Shows, T. B. Mucolipidosis II and III. The genetic relationships between two disorders of lysosomal enzyme biosynthesis. *J. Clin. Invest.* **72**, 1016–1023 (1983).
9 Little, L. E., Mueller, O. T., Honey, N. K., Shows, T. B. & Miller, A. L. Heterogeneity of *N*-acetylglucosamine-1-phosphotransferase within mucolipidosis III. *J. Biol. Chem.* **261**, 733–738 (1986).
10 Kudo, M., Brem, M. S. & Canfield, W. M. Mucolipidosis II (I-cell disease) and mucolipidosis IIIA (classical pseudo-hurler polydystrophy) are caused by mutations in the GlcNAc-phosphotransferase alpha/beta-subunits precursor gene. *Am. J. Hum. Genet.* **78**, 451–463 (2006).
11 Paik, K. H., Song, S. M., Ki, C. S., Yu, H. W., Kim, J. S., Min, K. H. *et al.* Identification of mutations in the GNPTA (MGC4170) gene coding for GlcNAc-phosphotransferase alpha/beta subunits in Korean patients with mucolipidosis type II or type IIIA. *Hum. Mutat.* **26**, 308–314 (2005).
12 Tiede, S., Storch, S., Lübke, T., Henrissat, B., Bargal, R., Raas-Rothschild, A. *et al.* Mucolipidosis II is caused by mutations in GNPTA encoding the alpha/beta GlcNAc-1-phosphotransferase. *Nat. Med.* **11**, 1109–1112 (2005).
13 Bargal, R., Zeigler, M., Abu-Libdeh, B., Zuri, V., Mandel, H., Ben Neriah, Z. *et al.* When mucolipidosis III meets mucolipidosis II: GNPTA gene mutations in 24 patients. *Mol. Genet. Metab.* **88**, 359–363 (2006).
14 Kojima, S., Okada, S., Kai, H., Ha, K., Nose, O., Ikeda, T. *et al.* A case of mucolipidosis II: biochemical, nutritional, and immunological studies. *Brain. Dev.* **1**, 26–30 (1979).
15 Okada, S., Owada, M., Sakiyama, T., Yutaka, T. & Ogawa, M. I-cell disease: clinical studies of 21 Japanese cases. *Clin. Genet.* **28**, 207–215 (1985).
16 Grewal, S., Shapiro, E., Braunlin, E., Charnas, L., Krivit, W., Orchard, P. *et al.* Continued neurocognitive development and prevention of cardiopulmonary complications after successful BMT for I-cell disease: a long-term follow-up report. *Bone Marrow Transplant.* **32**, 957–960 (2003).
17 Plante, M., Claveau, S., Lepage, P., Lavoie, E. M., Brunet, S., Roquis, D. *et al.* Mucolipidosis II: a single causal mutation in the *N*-acetylglucosamine-1-phosphotransferase gene (GNPTAB) in a French Canadian founder population. *Clin. Genet.* **73**, 236–244 (2008).

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RESEARCH REPORT

Validation of keratan sulfate level in mucopolysaccharidosis type IVA by liquid chromatography–tandem mass spectrometry

Shunji Tomatsu · Adriana M. Montaño · Toshihiro Oguma · Vu Chi Dung · Hirotaka Oikawa · Talita Giacomet de Carvalho · María L. Gutiérrez · Seiji Yamaguchi · Yasuyuki Suzuki · Masaru Fukushi · Luis Barrera · Tadao Orii

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Abstract Mucopolysaccharidosis type IVA (MPS IVA, Morquio A disease), a progressive lysosomal storage disease, causes skeletal chondrodysplasia through excessive storage of keratan sulfate (KS). KS is synthesized mainly in cartilage and released to the circulation. The excess storage of KS disrupts cartilage, consequently leaching more KS into circulation, which is a critical biomarker for MPS IVA. Thus, assessment of KS level provides a potential screening strategy and determines clinical course and efficacy of therapies. We have recently developed a tandem mass spectrometry [liquid chromatography (LC)/MS/MS] method to assay KS levels in blood. Forty-nine blood specimens from patients with MPS IVA [severe (n=33), attenuated (n=11) and undefined (n=5)] were analyzed for comparison of blood KS concentration with that of healthy subjects and for correlation with clinical severity. Plasma samples were digested by keratanase II to obtain disaccharides of KS. Digested samples were assayed by LC/MS/MS. We found that blood KS levels (0.4–26 μg/ml) in MPS IVA patients were significantly higher than those in age-matched controls (0.67–4.6 μg/ml; P<0.0001). It was found that blood KS level varied with age and clinical severity in the patients. Blood KS levels in MPS IVA peaked between 2 years and 5 years of age (mean 11.4 μg/ml). Blood KS levels in severe MPS IVA (mean 7.3 μg/ml) were higher than in the attenuated form (mean 2.1 μg/ml) (P=0.012). We also found elevated blood KS levels in other types of MPS. These findings indicate that the new KS assay for blood is suitable for early diagnosis and longitudinal assessment of disease severity in MPS IVA.

Abbreviations

KS	keratan sulfate
MPS IVA	mucopolysaccharidosis type IVA

Q1

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S. Tomatsu (✉) · A. M. Montaño · V. C. Dung · H. Oikawa · T. G. de Carvalho · M. L. Gutiérrez
Department of Pediatrics,
Saint Louis University Doisy Research Center,
1100 South Grand Blvd., Room 307,
St. Louis, MO 63104, USA
e-mail: tomatsus@slu.edu

T. Oguma
Daiichi-Sankyo Co., Ltd.,
Tokyo, Japan

S. Yamaguchi
Department of Pediatrics, Shimane University,
Matsue, Shimane, Japan

Y. Suzuki · T. Orii
Department of Pediatrics, Gifu University,
Gifu, Japan

M. Fukushi
Sapporo City Hygiene Institute,
Sapporo, Japan

L. Barrera
Instituto de Errores Innatos del Metabolismo,
Pontificia Universidad Javeriana,
Bogota, Colombia

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LSD	lysosomal storage disease
MPS	mucopolysaccharidoses
GAGs	glycosaminoglycans
GALNS	*N*-acetylgalactosamine-6-sulfate sulfatase
QC	quality control
CV	coefficient of variation
DMB	dimethylmethylene blue

Introduction

Mucopolysaccharidoses (MPS) are a family of heritable disorders caused by deficiency of lysosomal enzymes required for degradation of glycosaminoglycans (GAGs) (Neufeld and Muenzer 2001). Each known MPS type involves deficiency of a specific lysosomal enzyme required for the stepwise degradation of specific GAG(s).

Mucopolysaccharidosis type IVA (MPS IVA, Morquio A disease) is an autosomal recessive disease caused by the deficiency of *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS). The enzyme and its deficiency were discovered and identified by the use of oligosaccharide substrates prepared from chondroitin 6-sulfate (C6S) containing *N*-acetylgalactosamine (GalNAc) 6-sulfate. Thus, the enzyme was originally named GalNAc-6-sulfate sulfatase, abbreviated to GALNS (Dorfman et al. 1976). Subsequently, it was shown that the enzyme also removes the 6-sulfated galactose residues of keratan sulfate (KS) (Glössl and Kresse 1982). GALNS is required to degrade KS and C6S. Clinically, the severe form is characterized by short trunk dwarfism, kyphoscoliosis, coxa valga, odontoid hypoplasia, abnormal gait, joint mobility problems, restriction of chest wall movement and a life span of 20–30 years. Patients with the attenuated form can have a near normal quality of life, with mild involvement of the skeleton (Northover et al. 1996; Montaño et al. 2007, 2008). Although there is no approved treatment available, enzyme replacement therapy and bone marrow transplantation are under investigation on MPS IVA human patients and mouse models.

The pathogenesis of the bone dysplasia in MPS IVA is largely unknown, but it is speculated that the accumulation of KS could be toxic to osteoblasts (Fang-Kircher et al. 1997). A cost effective and sensitive method to measure a biomarker for the disease would be useful for making an early diagnosis, determining disease severity and systematically monitoring patients' responses to treatment regimens.

One potential disease marker for MPS IVA, total GAG in the urine, can be measured spectrometrically using dimethylmethylene blue (DMB) (Whitley et al. 1989) or alcian blue (Bjornsson 1993). However, these methods are not applicable to blood without prior protease treatment, as protein in the specimen interferes with the binding of the dye to the GAG. In addition, the dye itself is also prone to decompose, leading to a high background. For total GAG levels in urine, the range in approximately 20% of patients with MPS IVA overlapped the normal range (Tomatsu et al. 2004). Therefore it is difficult to distinguish the patients from healthy controls on the basis of the level of urine GAG excretion.

Another potential biomarker of this disease is the blood KS level. KS is synthesized mainly in cartilage and is one of the substrates accumulated in MPS IVA disease. The excessive storage of KS is known to cause severe skeletal dysplasia in patients with Morquio A syndrome. The measurement of KS in blood in these patients would provide information to help one to assess the longitudinal prognosis and efficacy of therapies, as well as early diagnosis. There are established procedures for measuring KS. The cetylpyridinium chloride method followed by thin-layer chromatography has been used semiquantitatively for the analysis of urine samples from patients with MPS IVA (Fujimoto and Horwitz 1983; Beck et al. 1986). Monoclonal antibody assay [enzyme-linked immunosorbent assay–inhibition assay (ELISA)-Inhibition] is also available for KS measurement (Thonar et al. 1985). However, those methods involve multiple laborious steps. We have recently reported a sandwich ELISA method for detection of KS in biological fluids. The results demonstrated that KS levels in the blood and urine of patients with MPS IVA are significantly elevated in comparison with those of healthy controls of equivalent ages (Tomatsu et al. 2004). The sandwich ELISA method provides the total amount of KS quantitatively but does not provide the level of sulfation and composition of KS. High-performance liquid chromatography (HPLC) is another sensitive and accurate method to measure each specific GAG, although it is still laborious and does not allow us to assay large numbers of samples simultaneously (Tomatsu et al. 2005; Kinoshita and Sugahara 1999; Toyoda et al. 1998; Yamada et al. 2000). We have recently developed a sensitive, specific and reproducible KS assay system by liquid chromatography tandem mass spectrometry (LC/MS/MS) (Oguma et al. 2007). We have also shown the importance of blood KS measurement to assess the efficacy of treatment during enzyme replacement therapy (ERT) on an MPS IVA mouse model (Tomatsu et al. 2008a), resulting in substantial reduction of KS level.

Moreover, we have recently demonstrated that KS level in blood is elevated in each type of MPS and mucolipidoses (ML) examined by sandwich ELISA, in contrast to the conventional understanding (Tomatsu et al. 2005).

In this study we evaluated KS levels in the blood of patients with MPS IVA by using a recently developed LC/MS/MS method, and we showed its feasibility as a method to evaluate the clinical course of MPS IVA. In addition, we assessed the elevation of KS levels in other types of MPS and ML.

Materials and methods

Subjects Blood (plasma) samples were obtained from 49 patients with MPS IVA (33 severe, 11 attenuated and five undefined) after informed consent had been obtained from each patient. For all samples, the ages of the patients were identified. Blood samples were also obtained from 125 healthy controls. The diagnosis of MPS IVA was made on the basis of a reduced enzyme activity (GALNS) of ≤5% the normal level in plasma, leukocytes or fibroblasts. We classified clinical severity according to patients' heights, as described previously (Montaño et al. 2008; Tomatsu et al. 2004). We obtained 101 blood (plasma) samples from patients with MPS and ML, except for those with MPS IV, ranging between 0 and 39 years of age (MPS I, n=31; MPS II, n=28; MPS III, n=19; MPS VI, n=6; MPS VII, n=6; ML, n=11).

KS assay Briefly, LC/MS/MS was used for the analysis of the disaccharides produced from KS. An API 4000 mass spectrometer equipped with a turbo-ion spray was used (Applied Biosystems, Foster City, CA, USA) (Oguma et al. 2007). KS in human plasma was digested to disaccharides by keratanase II (Seikagaku Corporation, Tokyo, Japan). The disaccharides were analyzed by LC/MS/MS using multiple reaction monitoring in negative ion mode. Separation by LC was performed on a Hypercarb column [2.0 mm internal diameter (i.d.)×150 mm, 5 μm) with gradient elution by acetonitrile–0.01 M ammonium bicarbonate (pH 10). The flow rate of the mobile phase was 0.2 ml/min. After digestion of the blood, KS was digested with keratanase II, and the disaccharide compositions of Galβ1→4GlcNAc(6S) and Galβ1(6S)→4GlcNAc(6S) were recognized. The C-6 position of the GlcNAc residue or both the Gal and GlcNAc residues was sulfated. When GALNS enzyme is deficient, the 6-sulfated galactose residues of KS are not removed in patients with MPS IVA; therefore, an accumulation of Galβ1(6S)→4GlcNAc (6S) disaccharides was expected. Blood samples with KS concentrations were assayed in duplicate using the appropriate dilution.

Data analysis The data obtained were analyzed to determine whether the levels of KS varied significantly with respect to age and clinical phenotype of MPS IVA patients. The variability of KS levels in the blood of both MPS IVA patients and controls was plotted according to age. For comparison between patient and control samples, Student's t-test, the Mann–Whitney U test or Welch's t-test was applied, depending on the distribution of the values. All data were analyzed with StatView statistical software (StatView J 4.5; Abacus Concepts, Inc.).

Results

Assay validation The intra- and inter-day precision and recoveries of Galβ1→4GlcNAc(6S) or Galβ1(6S)→4GlcNAc(6S) were previously obtained for human plasma and serum (Oguma et al. 2007). The results indicated that the method here had satisfactory precision and recovery without any effect by plasma proteins.

Blood (plasma) KS concentrations The KS values for the blood (plasma) samples from 49 MPS IVA subjects (average age 14.3 years; range 2–65 years), 125 control subjects (average age 12.1 years; range 0–62 years), and 101 non-type IVA MPS and ML are reported in Tables 1, 2 and 3 and Figs. 1 and 2.

Blood KS concentrations were found to vary with age. For all ages, the average blood KS concentrations in healthy controls and patients with MPS IVA were 2.3 μg/ml and 5.7 μg/ml, respectively (P<0.001). In healthy control newborn infants, blood KS concentration was under 2.0 μg/ml, and it rose, reaching a peak between the ages of 0 and 2 years (mean 2.9 μg/ml for control), and the concentrations stayed relatively constant till the individual reached 15 years of age. After 15 years, blood KS concentrations decreased to1.5 μg/ml and stabilized thereafter. When control subjects and MPS IVA patients in each age range (2–5 years, 5–10 years, 10–15 years, and over 15 years) were compared, the blood concentrations of KS were different between groups (mean 2.6 μg/ml vs 11.4 μg/ml, P<0.001; 2.2 μg/ml vs 6.4 μg/ml, P=0.055; 2.9 μg/ml vs 3.2 μg/ml, P=0.81; 1.5 μg/ml vs 2.1 μg/ml, P= 0.020) (Tables 1 and 2, Fig. 1). Thus, at younger ages, there was more difference in KS levels between MPS IVA patients and the age-matched controls. KS level in patients with MPS IVA was reduced and had nearly normalized at over 10 years old. Blood KS levels in severe MPS IVA (mean 7.3 μg/ml) were higher than in the attenuated form (mean 2.1 μg/ml) (P=0.012) (Table 2). This finding suggests that KS level could be associated with clinical severity (KS is metabolized), although it still remains noteworthy that the patients with the attenuated form had a lower mean KS because of their older age compared with patients with the severe phenotype.

The level of plasma KS was also compared between each type of MPS and ML and the age-matched controls (Tables 1 and 3 and Fig. 2). Plasma KS levels in MPS I showed that 4 out of 31 (12.9%) patients had above the mean+2 standard deviations (SD) of the age-matched controls (Fig. 2). Patients with MPS II had the highest mean KS in their blood among all types of MPS and ML patients except for patients with MPS IV (mean 6.0 μg/ml). Twenty-two out of 28 (78.6%) had plasma KS values above the mean + 2SD of age-matched controls (Fig. 1). Plasma